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Antibodies to a Soluble Form of a Tumor Necrosis Factor (TNF) Receptor Have TNF-like Activity*

(Received for publication, September 15, 1989)

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Immunological cross-reactivity between tumor necrosis factor (TNF) binding proteins which are present in human urine (designated TBPI and TBPII) and two molecular species of the cell surface receptors for TNF is demonstrated. The two TNF receptors are shown to be immunologically distinct, to differ in molecular weight (58,000 and 73,000), and to be expressed differentially in different cells. It is further shown that polyclonal antibodies against one of the TNF binding proteins (TBPI) display, by virtue of their ability to bind the TNF receptor, activities which are very similar to those of TNF. These antibodies are cytotoxic to cells which are sensitive to TNF toxicity, induce resistance to TNF toxicity, enhance the incorporation of thymidine into normal fibroblasts, inhibit the growth of chlamydiae, and induce the synthesis of prostaglandin E_2. Monovalent F(ab) fragments of the polyclonal antibodies lack TNF-like activities, but acquire them upon cross-linking with anti-F(ab)$_2$ antibodies, suggesting that the ability of the anti-TBPI antibodies to mimic TNF correlates with their ability to cross-link the TNF receptors. This notion was further supported by data obtained in a comparative study of the TNF-like cytotoxicity of a panel of monoclonal antibodies against TBPI.

The induction of TNF-like effects by antibodies to a TNF receptor suggests that TNF is not directly involved in intracellular signalling. Rather, it is the receptors to this cytokine which, when properly triggered in a process which appears to involve clustering of these receptors, transduce the signal for response to TNF into the cell's interior.

Two different concepts of the nature of tumor necrosis factor (TNF)[1] have directed the study of its mechanism of action. On the one hand, since the only known *in vitro* activity of TNF before its isolation was its ability to kill certain tumor cells (1–3), there has been a tendency to relate to TNF as a "killer" molecule. Accordingly, it has been suggested that TNF has an intrinsic destructive function (6–8) perhaps similar to that of some macrophage-produced proteins shown to have cell-killing enzymatic activities (4, 5). Alternatively, already in early studies on TNF, it was proposed that this cytokine may have no activity of its own and that, rather, it exerts its effects via activation of cellular mechanisms, eventually leading to cell death (7, 8). Findings reported after the isolation of TNF supported the latter concept. It was then clarified that TNF not only kills cells, it has many other effects related to various aspects of the inflammatory response and that, like other cytokines, TNF binds to specific cell surface receptors. By inference from knowledge of the mode of action of other polypeptide cytokines, it was assumed that the signalling mechanisms for the cellular response to TNF cannot reside in this protein itself; rather, they should be expressed by the receptors to which TNF binds and by cellular components associated with them. In the present study, we provide evidence for this notion and show that effects that are characteristic of TNF can be mimicked with antibodies which bind to the TNF receptors. These antibodies were raised against two TNF-binding proteins which had been isolated from human urine (9–11) and are immunologically cross-reactive with two molecular species of the TNF receptors (the present study and Ref. 12). The induction of TNF-like effects by antibodies reacting with one of the receptor species is shown to correlate with their ability to cross-link the receptor molecules, implying that the mere clustering of these receptors, even in the absence of TNF, provides a signal sufficient to induce effects which are characteristic of this cytokine.

* This work was supported by a grant from Inter-Lab Ltd., Nes-Ziona, Israel as well as by grants from the National Council for Research and Development, Israel, the German Cancer Research Center, and the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Cells

Cells of the SV80 (13) and HeLa (14) lines and human foreskin fibroblasts, strain FS11, were cultured in Dulbecco's modified Eagle's medium, HEp-2 cells (15) in minimal essential medium, and cells of the human histiocytic lymphoma line U937 (16) in RPMI 1640 medium. All media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

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Cytokines

Recombinant human TNF-α (rhu TNF-α 6 × 10^7 units/mg of protein), produced by Genentech Co., San Francisco, CA, was kindly provided by Dr. G. Adolf, of the Boehringer Institute, Vienna, Austria. Recombinant human interleukin 1α (rhuIL-1α, 3 × 10^7 leukocyte activating factor units/mg of protein), consisting of the 154 carboxyl-

terminal amino acids of the 271-amino acid human IL-1 precursor, was a gift of Drs. A. Stern and P. T. Lomedico (Hoffmann-La Roche), and recombinant human interferon-γ (rIFN-γ, 5×10^7 units/mg of protein) was a gift from Dr. D. Novick of our department.

Radiolabeling of TNF and TBPI

Both proteins were labeled with ^{125}I by the chloramine-T method as previously described (17). Specific radioactivities were 120 μCi/μg of protein for rhu TNF-α and 230 μCi/μg of protein for TBPI.

Rabbit Antisera

Purification of the TNF-binding proteins TBPI and TBPII from human urine and immunization of rabbits with the purified proteins were performed as described elsewhere (12). The titers of both antisera, to TBPI (ā TBPI) and TBPII (ā TBPII), as quantitated by determining the dilution at which they inhibit by 50% the binding of ^{125}I-TNF to HeLa and U937 cells, respectively, were about 1:6400. Rabbit antiserum to TNF-α was kindly provided by Dr. D. Maennel, of the German Cancer Research Center, Heidelberg, Federal Republic of Germany, and the antiserum to the IFN-γ receptor was a gift from Dr. D. Novick of our Institute. Rabbit anti-mouse immunoglobulin antiserum was purchased from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, PA). Goat anti-rabbit immunoglobulin antiserum was obtained from Biomakor (Israel).

Preparation of Monovalent F(ab) Fragments of the Rabbit Antibodies to TBPI

The immunoglobulins were purified from the ā TBPI antiserum by ammonium sulfate precipitation (at 50% saturation) followed by anion exchange high performance liquid chromatography (HPLC) on a Mono Q column (Pharmacia, Uppsala, Sweden; elution was accomplished using a gradient of 0–500 mM NaCl in 10 mM sodium borate buffer, pH 9.0, containing 0.02% sodium azide).

Digestion of the purified immunoglobulins with papain (twice crystallized, obtained from Sigma) (18) was carried out in the presence of 1 mM cysteine and 2 mM EDTA at an enzyme/substrate ratio of 1:100. The reaction was terminated by adding p-chloromercuribenzoate to a final concentration of 1 mM. The monovalent F(ab) fragments were purified by cation exchange HPLC on a Mono S column (Pharmacia LKB Biotechnology Inc.) (with a gradient of 0–300 mM NaCl in 10 mM sodium acetate buffer, pH 5.5, containing 0.02% azide). Purity of the immunoglobulins and of their monovalent F(ab) fragments was verified by SDS-PAGE analysis under both reducing and nonreducing conditions.

Monoclonal Antibodies to TBPI

Production of the Antibodies—BALb/c mice (8 weeks old, female) were injected four times with 1 μg of purified TBPI. The protein was first injected into the hind footpads in the form of an emulsion in complete Freund adjuvant. Three weeks later, the animals were injected subcutaneously in the back with TBPI in incomplete Freund adjuvant. The following two injections of TBPI in phosphate-buffered saline (PBS) were given subcutaneously in weekly intervals. Final boosts consisting of 9.0 μg of TBPI in PBS were given 4 days (intraperitoneally) and 3 days (intravenously) before fusion, which was performed as described before (19), using NSO cells (20) and lymphocytes prepared from both the spleen and local hind leg lymph nodes as fusion partners. Hybridomas were selected in Dulbecco's modified Eagle's medium supplemented with hypoxanthine/aminopterin/thymidine medium, 15% horse serum, and gentamycin (2 μg/ml). Those that were found to produce antibodies to TBPI were subcloned by the limiting dilution method and injected into BALb/c mice that had been primed with Pristane (2,6,10,14-tetramethylpentadecane, Aldrich) for the production of ascites. Immunoglobulins were isolated from the ascites by ammonium sulfate precipitation (at 50% saturation) and then dialyzed against PBS containing 0.02% azide. Purity was approximately 60%, as estimated by analysis on SDS-PAGE and staining with Coomassie Blue. The isotypes of the antibodies were determined using a commercially available enzyme-linked immunosorbent assay isotyping kit (Amersham).

Inverted Radioimmunoassay for the Detection of the Antibodies—This assay was used for estimating the level of anti-TBPI antibodies in the sera of the immunized mice and to screen for hybridomas producing such antibodies. Polyvinyl chloride 96-well microtiter plates (Dynatech 1-220-25) were coated for 12 h at 4 °C with affinity-purified goat anti-mouse F(ab) immunoglobulins (Biomakor, Israel; 10 μg/ml in PBS containing 0.02% NaN_3), then blocked for 2 h at 37 °C with 0.5% bovine serum albumin in PBS supplemented with 0.05% Tween 20 (Sigma) and 0.02% NaN_3 (blocking buffer) and washed three times with PBS containing 0.05% Tween 20 and 0.02% NaN_3 (washing buffer). Samples of serum or of hybridoma growth medium (50 μl) were incubated in the wells for 2 h at 37 °C. The plates were then rinsed with washing buffer, and ^{125}I-labeled TBPI (100,000 cpm, in blocking buffer) was placed in each well. After further incubation for 2 h at 37 °C, the plates were washed and the amount of label bound to individual wells was determined using a γ-counter.

Epitope Mapping of TBPI by Cross-competition Analysis with Monoclonal Antibodies—Polyvinyl chloride 96-well microtiter plates were coated as described above, with purified mAbs to TBPI (25 μg/ml). Following rinsing and blocking, samples of ^{125}I-labeled TBPI (50,000 cpm/well) which had been preincubated for 2 h, at 37 °C with the same or a different monoclonal antibody to TBPI (at 1 μg/ml) were put into the wells; the plates were incubated overnight at 4 °C and washed, and the radioactivity bound to each well was determined by γ-counting. The results are expressed as percent of the control values (TBPI binding in the absence of competing mAbs).

Effect of the Antibodies on Binding of TNF to HeLa Cells—HeLa cells were seeded into 15-mm tissue culture plates at a density of 2.5×10^5 cells/well. After 24-h incubation at 37 °C, in an atmosphere of 95% air and 5% CO_2, the cells were transferred to ice, the growth medium was removed, and the antibodies, diluted in Dulbecco's balanced salt solution (PBS$^+$) containing 0.5% bovine serum albumin and 0.1% sodium azide (PBS/bovine serum albumin), were added to the cells for 2 h. The cells were then rinsed and tested for binding of TNF as described elsewhere (21).

Cross-linking of ^{125}I-TNF to Intact Cells

HeLa cells (5×10^6, detached by incubation with PBS containing 5 mM EDTA) or U937 cells (1.5×10^6) were washed two times with ice cold PBS/bovine serum albumin and resuspended in this solution (10 ml) containing 3 nM radiolabeled TNF for 2 h at 4 °C. The cells were then washed three times with PBS$^+$ and treated for 20 min at 4 °C with 1 mM concentration of the cross-linking agent bis(sulfosuccinimidyl)suberate (Pierce) in the same buffer. Cross-linking was stopped by adding Tris-HCl and glycin HCl (both to a final concentration of 100 mM) followed by three washes with PBS$^+$. The cells were then pelleted by centrifugation at $500 \times g$ for 10 min and extracted for 1 h at 4 °C using 2 ml of a solution containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM benzamidine (Sigma), and 1 mM-phenylmethylsulfonyl fluoride (Sigma). After centrifugation (at $30,000 \times g$ for 15 min), the cell extracts were divided into 4 equal portions, and a sample of antiserum was added to each at a dilution of 1:100, followed by incubation for 12 h at 4 °C. Precipitation was achieved by addition of 20 μl of protein A-Sepharose (Pharmacia). After a 30-min incubation, the Sepharose beads were washed three times with 10 mM phosphate buffer, pH 7.2, containing 1% Triton X-100 and 2 M KCl (1 ml) and then twice with PBS$^+$ (1 ml). The beads were resuspended in 20 μl of sample buffer containing 4% (w/v) SDS and 6% (v/v) β-mecaptoethanol and boiled for 2 min, and the supernatant was analyzed by SDS-PAGE (7.5% acrylamide) followed by autoradiography.

Determination of Bioactivities of the Antibodies to TBPI and of TNF Cytotoxic Activity

Cells were seeded 24 h prior to assay in 96-well microtiter plates (3×10^4 cells/well). The antibodies or TNF were applied in serial dilutions either in the presence or absence of cycloheximide (CHI) (25 μg/ml in the case of the HeLa cells and 50 μg/ml for the other cells). After an incubation period of 12 h for the HeLa cells and 16 h for all the others, cell viability was determined by the neutral-red uptake method (22, 23). Values are presented as the per cent ratio of the viability of cultures incubated with CHI alone (for cells tested in its presence) or without additives.

Induction of PGE_2 Synthesis—Cells were seeded in 96-well microtiter plates (5×10^4 cells/well). Ten h thereafter, TNF and the antibodies were applied in serial dilutions. After further incubation for 15 h at 37 °C, the cell growth medium was collected and replaced with fresh medium containing arachidonic acid (5×10^{-6} M). One h later, the medium was collected again. The PGE_2 content of the samples was determined by immunoassay as described previously (24).

Stimulation of Fibroblast Growth—Growth stimulation in fibroblasts due to TNF and the antibodies to TBPI was determined essentially as described by Vilček *et al.* (25). Human foreskin fibroblasts (strain FS11, passage 10–12) were seeded in 96-well microtiter plates (10^4 cells/well). Either TNF or the antibodies to TBPI were added after an 18-h incubation. The rate of thymidine incorporation into the cells after 3 days of further incubation was determined by adding [^{3}H]thymidine to the cells (1 μCi/well) and incubating them for another 16 h. The cells were then rinsed once with cold PBS and detached with trypsin, and the amount of label incorporated was determined by harvesting the cells onto glass fiber filters followed by liquid scintillation counting.

Inhibition of Chlamydial Growth—The effect of the antibodies to TBPI on the growth of *Chlamydia trachomatis* (L-434/Bu) in the HEp-2 cells was determined as previously described for the antichlamydial effect of TNF (26). The antibodies were applied to the HEp-2 cells, either alone or together with IFN-γ: first, 1 day before infection with chlamydiae and again, at the same concentrations, immediately after infection. The yield of chlamydiae 2 days after infection was determined using an immunoperoxidase assay for the chlamydial antigens and is expressed as inclusion forming units/ml.

RESULTS

Immunological Cross-reactivity between TNF-binding Proteins Found in Human Urine (TBPI and TBPII) and Two Cell Surface TNF Receptors—Antibodies against two proteins purified from human urine that specifically bind TNF had inhibitory effects on the binding of TNF to cells. The relative effectiveness of the antibodies against the two proteins varied, depending on the cell system (12). We have suggested that the two urinary proteins (TNF-binding proteins, TBPI and TBPII) constitute soluble forms of two molecular species of the cell surface receptors for TNF and that the two receptors which were recognized by these antibodies are expressed differentially in cells of different lines. To further test this notion, we checked whether the antibodies to the urinary TNF-binding proteins can be used to immunoprecipitate the TNF receptors. Tagging the TNF receptors in cross-linking experiments with radiolabeled TNF revealed that the receptors in the HeLa and the U937 cells differ in size (58 kDa in HeLa and 73 kDa in the U937 cells, forming TNF-receptor conjugates of 75 and 90 kDa, respectively) (Fig. 1). Immunoprecipitation of the tagged receptors with antibodies to TBPI and to TBPII could be demonstrated. However, consistent with the differing extent to which antibodies against the two urinary TNF-binding proteins inhibit the binding of TNF to HeLa and U937 cells, these antibodies also differed in their ability to immunoprecipitate the receptors from extracts of the HeLa and U937 cells. The TNF receptors extracted from the HeLa cells ("type I" receptors) were specifically precipitated with antibodies to TBPI; the receptors of U937 cells ("type II") were specifically precipitated with antibodies to TBPII (Fig. 1).

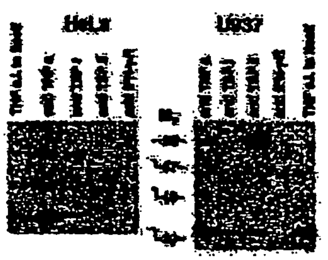

FIG. 1. Immunoprecipitation of TNF receptors from HeLa and U937 cells, tagged by chemical cross-linking to ^{125}I-TNF, using antibodies to TBPI and TBPII. The autoradiogram shows the analysis of proteins immunoprecipitated from Triton X-100 extracts of the indicated cells on a 7.5% polyacrylamide gel. Binding of radiolabeled TNF to these cells, cross-linking with bis(sulfosuccinimidyl)suberate and immunoprecipitation with rabbit antisera (to TNF, TBPI, TBPII, and the receptor to IFN-γ, all at a dilution of 1:100) was performed as described under "Materials and Methods." For comparison, ^{125}I-labeled TNF was cross-linked to itself (*TNF c.l. to itself*) and analyzed on the same gel. The molecular weight of the cross-linked products was determined by comparison to a ^{14}C-labeled marker set, purchased from Amersham.

Using a panel of monoclonal antibodies (mAbs) to TBPI, we attempted to analyze the extent of the immunological cross-reactivity between TBPI and the type I cell surface TNF receptor. Epitope mapping of TBPI by cross-competition analysis with 7 mAbs suggested that these antibodies bind to four topologically distinct areas on the molecule (*A*, defined by mAbs 17 and 23; *B*, by 18; *C*, by 20 and 34; and *D*, by 30 and 68 (Fig. 2)). An exceptional competition pattern was observed for the antibodies associated with epitope region D. The antibodies not only competed effectively with each other but also with the antibodies belonging to regions B and C. Moreover, the antibodies defining regions B and C competed, although less effectively, with the antibodies which bind to epitope D. One possible interpretation for this phenomenon is that epitopes B and C, while spatially distinct, both overlap with the epitope region represented by group D. An alternative possibility is that binding of an antibody to the epitope region D imposes a conformational change on TBPI which prevents the binding of the antibodies recognizing determinants in B and C. The latter hypothesis seems consistent with the fact that the antibodies defining epitope D were the only ones to recognize TBPI after its denaturation with SDS in the presence of β-mercaptoethanol (not shown).

All the monoclonal antibodies against TBPI (the 7 described in Fig. 2 and 10 others) had marked inhibitory effects on the binding of TNF to TBPI (not shown) as might be expected considering the molecular size difference between immunoglobulins and TBPI. All the antibodies also had an inhibitory effect on TNF binding to HeLa cells (Fig. 2 and data not shown), suggesting that the different epitopes which they recognize in the TBPI molecule are also present in the cell surface receptors for TNF.

Antibodies to TBPI Have TNF-like Effects—Applying the polyclonal antibodies to TBPI on cells in the presence of the protein synthesis inhibitor cycloheximide (CHI) resulted, within a few hours, in extensive cytolysis. The cytocidal effect was complement-independent (data not shown) and appeared to be morphologically very similar to the cytocidal effect of TNF. Resemblance to effects of TNF in several other respects were also found.

(*a*) The sensitivity of different cell lines to polyclonal anti-TBPI antibodies (& TBPII) and to TNF followed similar patterns. Thus, human foreskin fibroblasts (strain FS11) and HEp-2 cells, which are relatively resistant to TNF toxicity, showed also a low sensitivity to the toxicity of the antibodies (Fig. 3).

(*b*) Like TNF, the antibodies failed to kill HeLa and SV80 cells in the absence of protein synthesis inhibitors (see legend to Fig. 3).

(*c*) The sensitizing effect of protein synthesis inhibitors to the antibody-mediated cell killing was largely dependent on the timing of their application. Maximal cytotoxity could be observed when the inhibitors and the antibodies were applied simultaneously. Application of the inhibitors a few hours after the antibodies resulted in significantly less cell death (Table I). The same time dependence was observed for the sensitization by such inhibitors to the cytocidal effect of TNF ((27, 28) and Table I). Thus, the antibodies, like TNF, could be either cytotoxic to cells or induce in them resistance to their

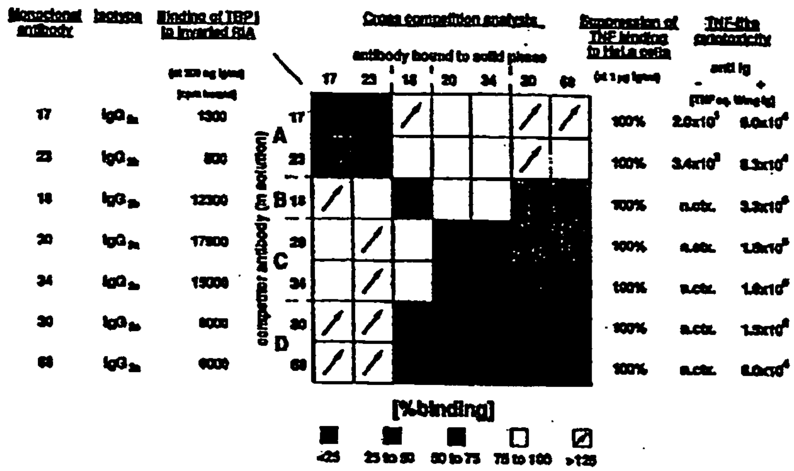

FIG. 2. **Epitope mapping of TBPI by cross-competition analysis with different mAbs and correlation with the ability of the antibodies to suppress binding of TNF to HeLa cells and to mediate TNF-like cytotoxicity.** Binding of radiolabeled TBPI to the mAbs (applied at a saturating concentration, 200 ng/ml) in the inverted radioimmunoassay, cross-competition analysis of the mAbs for their binding to TBPI and determination of the effect of the mAbs on the binding of TNF to HeLa cells were carried out as described under "Materials and Methods." The TNF-like cytotoxicity of the antibodies was measured in two ways. In the first, (*−Ig*), SV80 cells were incubated for 16 h at 37 °C with the mAbs in the presence of CHI (50 μg/ml). In the second, (*+anti Ig*), the effect of cross-linking by anti-immunoglobulin antibodies on the cytotoxity of the mAbs was tested. In this case, the SV80 cells were pulse-treated with the antibodies for 2 h at 4 °C, rinsed, and further incubated for 16 h at 37 °C with rabbit anti-mouse F(ab)$_2$ antibodies (*+anti Ig*) in the presence of CHI. Cell viability was determined as described under "Materials and Methods." The cytotoxic activity of the antibodies is expressed in TNF equivalent units per mg of immunoglobulin where 1 TNF equivalent unit is defined as the amount of antibody exerting the same cytotoxicity as 1 unit/ml TNF (16 pg/ml) (*n.ctx.*, not cytotoxic).

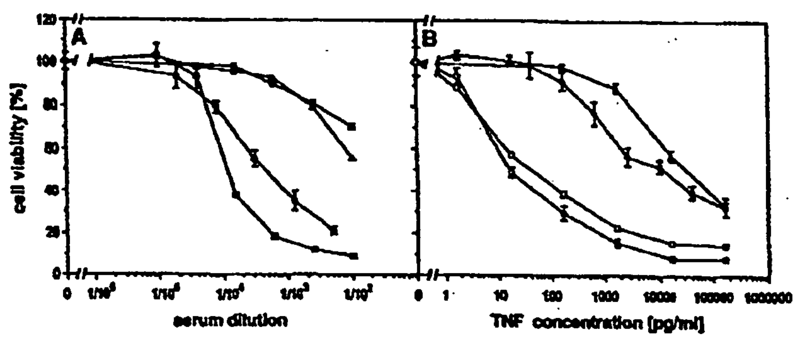

FIG. 3. **The cytocidal effect of the antibodies to TBPI (*A*) and of TNF (*B*) on SV80 (○, ●), HeLa (□, ■), FS11 (△, ▲), and HEp-2 (◇, ◆) cells.** The antibodies and TNF were applied for 16 h (12 h for the HeLa cells) together with CHI (25 μg/ml for HeLa and 50 μg/ml for all other cells). Cell viability was quantitated by measuring the uptake of neutral red dye. Viability of cells incubated with anti-TBPI at 1:200 in the absence of CHI was 99% in the SV80 cells, 97% in HeLa cells, 98% in FS11 cells, and 96% in the HEp-2 cells. Normal rabbit serum in the range of concentrations of ã TBPI applied in this study had no effect in this experiment nor in any of the other experiments presented below. All tests were performed in duplicate.

own toxicity, depending on whether they were applied in the presence or absence of protein synthesis inhibitors.

(*d*) Resistance to the toxicity of both the antibodies and of TNF was induced in the SV80 cells also by pretreatment with IL-1 (Table II, see also Ref. 28). Furthermore, the antibodies and TNF could induce in these cells cross-resistance to each other's toxicity (Table II).

Further examination of the effect of antibodies to TBPI, when applied on cells in the absence of protein synthesis blockers, revealed that under these conditions the antibodies mediate several noncytocidal TNF-like effects. In the foreskin fibroblasts and HEp-2 cells, which are quite resistant to TNF cytotoxity, as well as in the TNF-sensitive HeLa cells, the antibodies, similarly to TNF (29), had a marked stimulatory effect on the synthesis of prostaglandin E_2 (Fig. 4). In both cases, effects were particularly prominent when arachidonic acid was added to the cells, suggesting that it reflects an increase, not in the release of arachidonic acid, but in its conversion to prostaglandin. An additional TNF-like effect of the antibodies in the foreskin fibroblasts was enhancement of thymidine incorporation (Fig. 5), apparently reflecting stimulation of cell growth. Like the growth-stimulatory effect of TNF, the stimulation of fibroblast growth by the antibodies was obliterated when the cells were treated simultaneously with IFN-γ (Fig. 5).

In HEp-2 cells, TNF suppresses the growth of chlamydiae, obligate parasitic bacteria which grow intracellularly within membrane-bound structures (26). As shown in Fig. 6, growth

TABLE I

Time-dependent sensitization of SV80 cells to the cytocidal effect of TNF or antibodies to TBPI, by CHI

SV80 cells were incubated for 16 h with TNF or the antibodies to TBPI. CHI (50 μg/ml) was added to the culture at time zero or at 1, 3, or 6 h after application of TNF or the antibodies. Cell viability was determined at the end of the incubation period by the neutral red uptake method.

Time of CHI application	Cell viability	
	TNF (100 units/ml)	ā TBPI (1:200)
	%	
Simultaneously with ā TBPI/TNF	<1	<1
+1 h	<1	<1
+3 h	41	48
+6 h	77	73
Not added	100	100

TABLE II

Induction of resistance to the cytocidal effects of ā TBPI or TNF by TNF itself, ā TBPI, and IL-1

SV80 cells were treated for 1 h with TNF, IL-1, ā TBPI or without additives and incubated further for 6 h in medium alone to allow full recovery of the TNF receptors (pretreatment). The cells were then incubated for 12 h with TNF or ā TBPI in the presence of CHI or with CHI alone (treatment). While without any pretreatment most cells were killed when incubated with TNF or ā TBPI together with CHI, cells which were first incubated with TNF, IL-1, or ā TBPI in the absence of CHI were largely resistant to subsequent treatment with TNF or ā TBPI in the presence of CHI.

Treatment		Pretreatment (for 6 h)		
		TNF (100 units/ml)	IL-1 (10 units/ml)	ā TBPI (1:200)
		% cell viability		
CHI (50 μg/ml)	100	91	95	85
TNF (10^4 units/ml) + CHI	8	82	82	71
ā TBPI (1:200) + CHI	9	89	84	ND[a]

[a] ND, not determined.

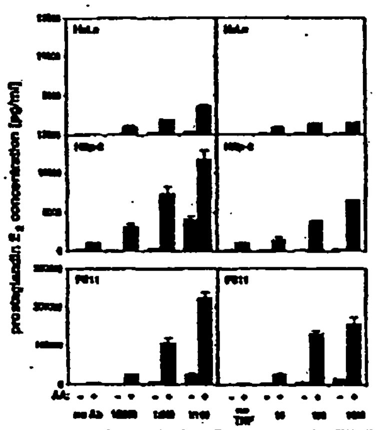

FIG. 4. **Enhancement of PGE_2 synthesis by the antibodies to TBPI and by TNF in HeLa, HEp-2, and FS11 cells and its augmentation by arachidonic acid (*AA*, 50 μM). For details, see "Materials and Methods."**

of chlamydiae in these cells was also markedly inhibited by the antibodies to TBPI. Inhibition of chlamydial growth by TNF is synergistic with the antichlamydial effect of IFN-γ (26) and is largely abrogated when the HEp-2 cells are grown in the presence of increased concentrations of tryptophan (30). The antichlamydial effect of the antibodies to TBPI was affected by IFN-γ and tryptophan in a similar manner (Fig. 6).

The TNF-like Activity of the Antibodies to TBPI Correlates with Their Ability to Cross-link the TNF Receptor Molecules— To explore the mechanisms for the TNF-like activity of antibodies to TBPI, we tested the effect of monovalent F(ab) fragments of ā TBPI on cell function. Like the intact antibodies, the monovalent fragments effectively blocked the binding of radiolabeled TNF to cells, suggesting that they maintained the ability to bind to the cell surface TNF receptors (EC_{50} was about 0.8 μg/ml for the intact antibodies and 1 μg/ml for the monovalent fragments). However, while in their intact form the antibodies were cytotoxic to CHI-treated SV80 cells at concentrations as low as 0.1 μg/ml, the monovalent F(ab) fragments of the antibodies did not exhibit toxic effects (Fig. 7*A*). Indeed, by virtue of their ability to inhibit the binding of TNF to cells, the monovalent F(ab) fragments not only failed to kill the SV80 cells but even had some inhibitory effect on their killing by TNF (Fig. 7*C*).

To check whether this loss of TNF-like activity in the fragmented antibodies was related to their monovalence, we investigated whether cross-linking of the F(ab) fragments would result in resurgence of their cytotoxic activity. It had been shown previously that pulse treatment of SV80 cells with TNF at 4 °C, followed by incubation with CHI at 37 °C, is sufficient to cause cell death (31). The intact antibodies to TBPI were also cytotoxic under these conditions (compare *solid* and *empty circles* in Fig. 7*A*), while monovalent F(ab) fragments were not cytotoxic. However, when the F(ab)-pretreated cells were treated subsequently with goat antibodies to rabbit Ig to elicit cross-linking of the cell-bound antibody fragments, extensive cell death occurred (Figs. 7*B* and 8).

To further study the molecular requirements for triggering TNF-like biological activities with antibodies to TBPI, we checked whether mAbs against TBPI mediate TNF-like cytotoxicity. Two experimental approaches were taken: in the first, the antibodies were tested for cytotoxicity to CHI-treated SV80 cells without any further treatment (Fig. 2, *−anti Ig*) and in the second, the cytotoxicity of the mAbs was tested after cross-linking them with anti-mouse immunoglobulin antibodies (Fig. 2, *+anti Ig*). As noted above, all mAbs tested appeared to bind to the type I TNF receptors. However, only 2 of the 17 mAbs had mild cytotoxic activity on SV80 cells (Fig. 2 and data not shown). This difference in biological activity among the mAbs could not be correlated with their isotype nor with their binding capacity in the inverted radioimmunoassay (Fig. 2). It did appear, however, to relate to the binding site of the antibodies on the receptor molecule. In cross-competition analysis, the two cytotoxic antibodies were found to bind to the same epitope region in TBPI (*A* in Fig. 2), whereas none of the other antibodies bound to it. In retesting the effect of the antibodies after cross-linking them with anti-immunoglobulin antibodies, we found them all to be highly cytotoxic, to an extent which appeared roughly proportional to the effectiveness with which they bound TBPI (compare in Fig. 2: *cytotoxity + anti Ig to the TBPI binding in inverted RIA*).

In testing whether different mAbs to TBPI can supplement each other in mediating TNF-like cytotoxicity, we found that mixtures of two mAbs mapping to different epitope regions on TBPI were highly cytotoxic to SV80 cells. For example, mixtures of the mAb 18 (epitope region B), which is not cytotoxic by itself, with the noncytotoxic mAbs 20 or 34 (both epitope region C), exerted strong cytotoxicity (approximately

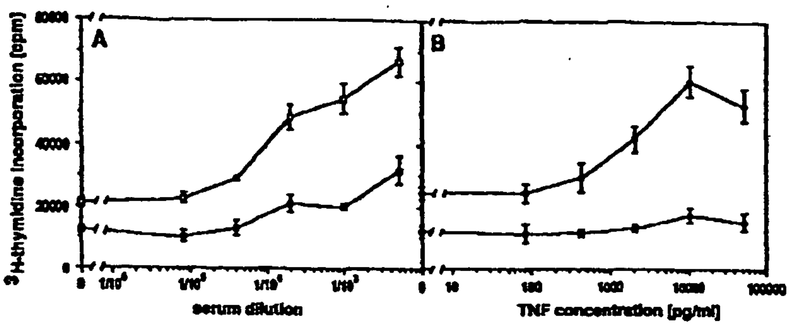

FIG. 5. Growth-stimulatory effect of the antibodies to TBPI (*A*) and of TNF (*B*) on human fibroblasts and its reversion by IFN-γ. Human foreskin fibroblasts (strain FS11) were incubated for 3 days with the antibodies to TBPI (□) or with TNF (O), in the presence (■, ●) or absence (□, O) of IFN-γ (250 units/ml). At the end of this incubation period, the rate of [^{3}H]thymidine incorporation was determined as described under "Materials and Methods."

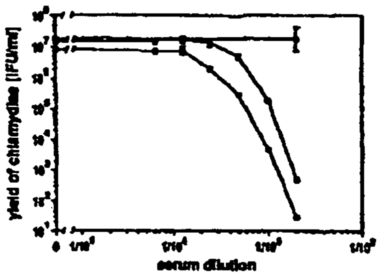

FIG. 6. The antichlamydial effect of the antiserum to TBPI, enhancement of the effect by IFN-γ, and its abolition at a high tryptophan concentration. The effect of the antiserum, at the indicated dilutions, in the presence (●) or absence (O) of IFN-γ (2 units/ml) was quantitated as described under "Materials and Methods." Increase of tryptophan concentration (at the time of infection of the cells treated with ā TBPI) from 10 μg/ml to 200 μg/ml abolished the antichlamydial effect (▲).

3×10^5 TNF equivalent units/mg of Ig; see Fig. 2 for definition of units). Mixtures of two mAbs mapping to the same epitope region were not more cytotoxic than each mAb alone.

DISCUSSION

Findings presented in this study provide further evidence for the immunological cross-reactivity between cell surface receptors for TNF and the two TNF-binding proteins found in human urine; they also demonstrate the use of antibodies against the soluble TNF-binding proteins as a technical aid in exploring the mechanism of TNF function.

The correlation demonstrated in this study between the efficacy of antibodies against TBPI and TBPII in suppressing the binding of TNF to its receptor in different cell lines and their ability to immunoprecipitate these receptors indicates the existence of two immunologically distinct TNF receptors which are differentially expressed in cells of different lines. As has been recently noted in another study (32), there is also a size difference between the two molecular species of TNF receptors. The estimated sizes, as measured by SDS-PAGE, are 58 kDa and 73 kDa for the receptors recognized by the antibodies to TBPI ("type I") and TBPII ("type II"), respectively.

Polyclonal antibodies to TBPI are highly cytotoxic to TNF-sensitive cells which express the type I receptors; moreover, they elicit several noncytocidal effects which are characteristic of TNF. Cell specificity of the response to the antibodies and the ways in which this response was modulated were very similar for TNF. Thus, we found differences in sensitivity to the cytotoxity of the antibodies among cells of different lines to be well-correlated with differences in their sensitivity to the cytocidal effect of TNF, as were induced variations in cell

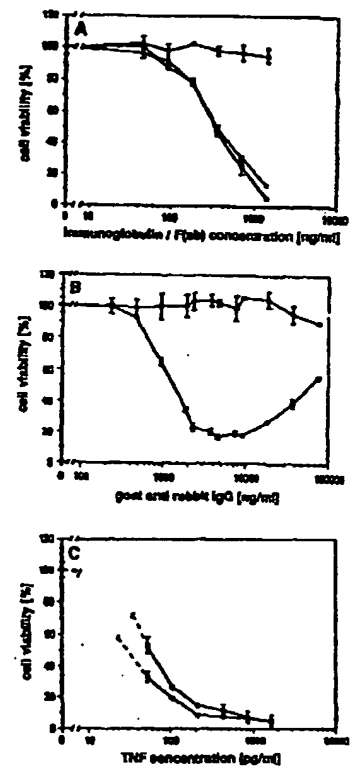

FIG. 7. Lack of cytocidal activity in monovalent F(ab) fragments of the antibodies to TBPI and recovery of that activity by cross-linking the F(ab) fragments with anti-immunoglobulin antibodies. *A*, the cytocidal effect of ā TBPI immunoglobulins (O) and of their monovalent F(ab) fragments (◊) at different concentrations, when applied to SV80 cells for 16 h together with CHI (50 μg/ml). Titration of the cytocidal effect of "pulse" treatment with the ā TBPI immunoglobulins (●) was performed as follows. The cells were incubated with the antibodies, at the indicated concentrations, for 2 h at 4 °C, and then rinsed and incubated at 37 °C for an additional 16 h with CHI (50 μg/ml) with no further addition of the antibodies. *B*, effect of goat antibodies to rabbit immunoglobulins when applied (at 5 μg/ml) to SV80 cells after pulse treatment with the monovalent F(ab) fragments of the ā TBPI (●) or to untreated cells (O). The pulse treatment with the F(ab) fragments was performed as described for the intact immunoglobulins in *A*. *C*, protection from TNF cytotoxicity by the monovalent fragments of the antibodies to TBPI: SV80 cells which were pulse treated with the F(ab) fragments, as in (*B*) (●), and, for comparison, cells treated in the same way with medium alone (O) were further incubated for 16 h with TNF, at various concentrations, together with CHI (50 μg/ml).

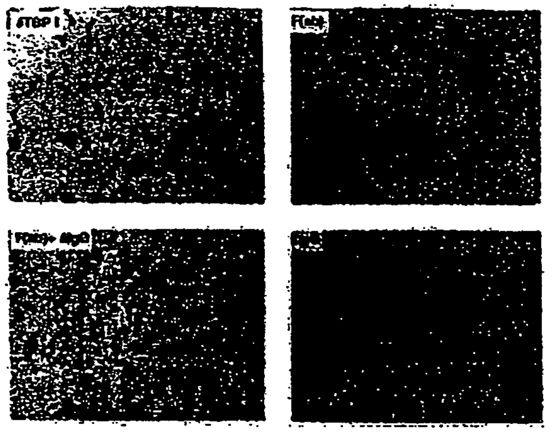

FIG. 8. Morphology of SV80 cells after pulse treatment with monovalent F(ab) fragments of a TBPI and further incubation in the presence or absence of anti-immunoglobulin antibodies. Anti-TBPI immunoglobulins and their monovalent F(ab) fragments were applied to the SV80 cells for 2 h in the cold, at a concentration of 8 μg/ml, followed by rinsing and incubation for 16 h at 37 °C in the presence of CHI with or without goat anti-rabbit IgG (5 μg/ml). All other conditions of the assay were as described in the legend to Fig. 7. Photographs were taken at a magnification of ×125 after staining the cells with neutral red.

response to TNF and the antibodies. Inhibition of protein synthesis by CHI sensitized cells to the cytocidal effect of the antibodies as also to that of TNF. In the absence of protein synthesis inhibitors, the SV80 cells responded to treatment with the antibodies, TNF or IL-1, with increased resistance to a subsequent challenge with the antibodies + CHI or with TNF + CHI. The modulation of noncytotoxic TNF-like activities of the antibodies by various agents, including IFN-γ, arachidonic acid, and tryptophan, also resembled the way these agents modulate the activities of TNF.

In all, these correlations strongly indicate that the functional alterations which the antibodies induce in cells are identical with those induced by TNF.

Studies on ligand-mimicking effects of antibodies to other receptors indicate two possible mechanisms for such effects. The antibody may stimulate the receptor by interacting with the binding site for the agonist, presenting to the receptor, as an internal image, a structure identical with that of the agonist. Alternatively, the activity of the antibodies may simply reflect their ability to cross-link the receptor molecules. Well known examples of the latter mechanism are the activation of mast cells upon aggregation of their Fc receptors in response to aggregated IgE (33), the stimulation of T cells by mitogenic lectins (34), and the induction of insulin-like effects by antibodies or lectins which interact with the insulin receptor (35).

Among the 17 monoclonal anti-TBPI antibodies tested, only two, 17 and 23, mediated TNF-like activity without further cross-linking them with anti-immunoglobulin antibodies. This activity was much weaker than the cytocidal effect observed when these antibodies were cross-linked with the use of anti-Ig antibodies. The fact that these two antibodies mapped both to the same epitope region and that this region (A, Fig. 2) is distinct from those to which all other antibodies were bound suggest that the first of the above two mechanisms is involved in their function. However, as has been proposed in a study on the IgE-like activity of monoclonal antibodies against the Fc receptors of mast cells, differences in ability of antibodies to exert an agonist-like effect may be just a reflection of differences in configurational restraints which these antibodies impose on the receptor molecule (36). Thus, the configurational restraints imposed on the TNF receptor by binding antibodies to epitope region A may be more favorable to stimulation of the receptors upon their dimerization than those imposed by binding of antibodies to other parts of the receptor molecule.

Regardless of whether or not the presence of a molecular structure resembling TNF is involved in the TNF-like activity of mAbs 17 and 23, several points make it clear that antibodies can mediate such TNF-like activity even when there is no such resemblance.

(*a*) Monovalent F(ab) fragments of the polyclonal antibodies to TBPI, even though still able to bind the TNF receptors, lack TNF-like activities.

(*b*) The monovalent fragments regain TNF-like activity when cross-linked with anti-immunoglobulin antibodies.

(*c*) Cross-linking with anti-immunoglobulin antibodies endows also the mAbs to TBPI with a potent cytocidal activity.

(*d*) The ability of cross-linked mAbs to mediate TNF-like cytotoxicity is independent of the epitope on the receptor molecule to which they bind.

(*e*) The efficacy with which antibodies to TBPI mediate TNF-like cytotoxicity is correlated with the extent of receptor aggregation they can cause. Polyclonal antibodies and mixtures of monoclonal antibodies against spatially distinct epitopes in the receptor, which potentially can cause massive aggregation of the receptor molecule, were much more effective than single mAbs, which at most can cause dimerization of the receptors.

The above observations suggest that aggregation of the TNF receptors, irrespective of the site on the TNF receptor to which the aggregating agent binds, is sufficient by itself to trigger a TNF-like effect.

It is likely that aggregation of receptors plays a role in the signalling mechanism, not only in activating receptors by artificial means, such as anti-receptor antibodies, but also in stimulating these receptors by their natural agonists. For example, evidence has been presented that the epidermal growth factor (EGF) receptors, which are stimulated when aggregated with anti-receptor antibodies (37), aggregate in response to EGF (38). In this connection, it is of interest to note that TNF exists in oligomeric forms, dimers and trimers, and that, in its monomeric form, TNF has little or no biological activity (39–42). This difference in activity between monomeric and oligomeric TNF was ascribed to a decreased affinity of the monomers to the TNF receptors (40). It is tempting to speculate that, in addition, the lower activity of TNF monomers reflects dependence of the function of TNF on clustering of the TNF receptors, and that it is the association between the TNF protomers which imposes the clustering of the receptors.

Because of our particular interest in the cytocidal effect which TNF mediates and in its initiation, and since the cells used in our prior studies of TNF-mediated cytotoxicity express, primarily, the type I receptors, we have focused on examining the effects which antibodies against TBPI can have on cells. It remains to be determined whether effects mediated by the type II receptors, which are immunologically cross-reacting with TBPII, can also be mimicked with anti-receptor antibodies. Moreover, it is not certain whether all effects mediated by the type I receptor are inducible by antibodies. However, it is clear from the data presented in this study that several different effects can be induced in this way, including some which are dependent on protein synthesis and at least one which is independent of it. Cell killing by

TNF occurs independently of protein synthesis. It is antagonized by mechanisms which do depend on the synthesis of proteins and which may themselves be enhanced by TNF (23, 27). Both the protein synthesis-independent, cytotoxic effect and the protein synthesis-dependent, induced increase in resistance to TNF toxicity could be mimicked by the antibodies to TBPI. Induced proteins are also involved in other TNF effects which these antibodies mimicked. Increased PGE_2 synthesis in response to TNF can be blocked by protein synthesis inhibitors and is apparently mediated by an increase in the enzyme prostaglandin-endoperoxide synthase (43). The antichlamydial effect of TNF involves the function of IFN-γ and of enzyme(s) which degrade tryptophan, perhaps indoleamine 2,3-dioxygenase (30). The mechanisms for the growth-stimulatory effect of TNF in fibroblasts are not known, but the effect was shown to be correlated with enhanced synthesis of epidermal growth factor receptors (44).

Induction of TNF-like effects in the absence of TNF itself may not necessarily be restricted to the *in vitro* conditions defined in the present study. One obvious *in vivo* situation in which this phenomenon may well occur is in autoimmune disorders, where antibodies to a variety of self-antigens, including certain cell surface receptors, are produced (45). It would be of great interest to ascertain whether, in any such disease, autoantibodies to the TNF receptors or to their soluble forms are formed, and to define the extent to which these antibodies, by mimicking the effects of TNF, may contribute to the pathogenesis of the disease. The finding that effects characteristic of TNF can be induced in its absence may have even further bearing on their physiological and pathophysiological significance. Not only antibodies which bind to the TNF receptor, but also other agents, pathogenic, like components of viruses or bacteria, or physiological, such as cytokines whose receptors interact with that of TNF, may be able to perturb the structure of the TNF receptor in a way resulting in activation. It has been recently reported that a cytocidal effect, similar to that of TNF, can be elicited in certain cells by antibodies which bind to a cell surface protein which is distinct from the TNF receptors and yet appears to be associated with them (46). We may thus expect that the spectrum of physiological and pathological situations involving effects characteristic of TNF will turn out to be much wider than the range of situations in which TNF is actually present.

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